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Lack of binding of glyceraldehyde-3-phosphate dehydrogenase to erythrocyte membranes under in vivo conditions

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A filtration method is described for separating membrane-free cytoplasm from concentrated erythrocyte haemolysates. The method has been used to assess glyceraldehyde-3-phosphate dehydrogenase binding to erythrocyte membranes. The relative amounts of glyceraldehyde-3-phosphate dehydrogenase and lactate dehydrogenase in the cytoplasm (either oxygenated or deoxygenated) indicate there is no detectable binding of glyceraldehyde-3-phosphate dehydrogenase to the membranes under physiological conditions.

Introduction

There is considerable interest in whether there is compartmentation of soluble enzymes within cells, as either membrane bound entities or free multienzyme complexes. For the human red blood cell there is good evidence for the binding of certain glycolytic enzymes to the cytoplasmic surface of isolated membranes when the ionic strength is low [1–4]. However, in the intact cell under physiological conditions the evidence for significant enzyme binding is less directly obtained and more controversial. For glyceraldehyde-3-phosphate dehydrogenase (EC 1.2.1.12) an electron microscope study of the distribution of ^{3}H-labelled enzyme [5] and glutaraldehyde cross-linking experiments [6] showed the enzyme was concentrated close to the membrane in the intact cell. Although this work does not necessarily imply the enzyme is membrane bound it is apparently in agreement with the results of Kliman and Steck [7], who studied glyceraldehyde-3-phosphate dehydrogenase release from erythrocytes during haemolysis and concluded that the kinetics were described by a redistribution between free and bound states with about 65% of the enzyme being membrane bound at the onset of haemolysis. However, more recent work [8] has shown that free cytoplasmic contents are released relatively slowly during haemolysis and any extrapolation of the kinetics of enzyme release during haemolysis to zero time to determine the amount of bound enzyme in the intact cell, must take this incomplete haemolysis into account. When this is done, extrapolation to zero time indicates the amount of membrane-bound enzyme in the intact cell is very small.

This later work is open to the criticism that the erythrocyte at the point of haemolysis is not comparable to the in vivo state. An attempt to mimic this state was made by Simpson et al. (1983) in studying the binding of fructosebisphosphate aldolase (EC 2.7.1.11) [9] and glyceraldehyde-3-phosphate dehydrogenase [10] to erythrocyte membranes. These workers haemolysed packed erythrocytes and separated free from membrane-bound components by centrifugation. This approach has the advantage that the dilution of cytoplasm and stretching of the membrane by

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colloid osmotic pressure swelling is limited by the small volume of extracellular fluid available. However, the method suffers from the disadvantage that the centrifugation takes some hours, and for the glyceraldehyde-3-phosphate dehydrogenase binding studies was done at a non-physiological temperature (4°C). In studying the binding of glyceraldehyde-3-phosphate dehydrogenase to erythrocyte membranes we have adopted the approach of Simpson, but reduced the time involved in separating free from membrane-bound components by using a filtration method, which we carried out at 37°C.

Experimental

Materials and general methods

Fresh blood was obtained from healthy human donors by venipuncture. When haemolysates were to be prepared from fresh washed cells, heparin (10–20 units/ml of blood) was used as anticoagulant. For haemolysates from fresh unwashed cells the blood was defibrinated. This procedure was considered necessary because anticoagulants displace membrane-bound glyceraldehyde-3-phosphate dehydrogenase (Rich, G.T., unpublished work). Defibrination was done by a previously described method [11], except that the defibrinating tool was glass, not steel. Metabolically depleted cells were obtained from blood bank blood that had been stored in citrate/phosphate/dextrose at 0–4°C for 5 weeks.

Gas mixtures ($5\%CO_2/95\%O_2$ and $5\%CO_2/95\%N_2$) were from BOC Ltd. The gases were bubbled through 0.15 M NaCl before use. pO_2 and pH of deoxygenated haemolysates were measured using a Radiometer PHM 71 acid-base-gas analyser.

The sources of chemicals, enzymes and co-enzymes were as described previously [8]. Lactate dehydrogenase (EC 1.1.1.27), aldolase and acetylcholinesterase (EC 3.1.1.7) were assayed by the methods of Beutler [12]; glyceraldehyde-3-phosphate dehydrogenase was assayed by the modification of the method of Warburg and Christian [13] described in our earlier paper [8]. The occasional problem encountered and referred to in that paper, of some glyceraldehyde-3-phosphate dehydrogenase being incompletely accessible to substrates was overcome by including 0.01% saponin in the assay medium. K^+ was measured by flame photometry using a Corning 400 flame photometer.

25-mm diameter filters and prefilters were obtained from Millipore UK Ltd. (SSWP02500 and HAWG02500 for 3 μm and 0.45 μm pore size filters, respectively; AP2502500 for prefilters) and Sartorius (SM11104 for 0.8 μm pore size filters). Untreated filters released small amounts of K^+ into the filtrates. When K^+ was to be assayed the filters were washed with 0.15 M NaCl, and then with water until the conductivity of the washings returned to the value of the pure water. The filters were dried overnight either in a vacuum desiccator or at 40°C.

The filtrates and total haemolysates were diluted into an enzyme stabilizing solution containing 0.1 M Tris-HCl (pH 8.0), 2 mM NaEDTA, 0.1% saponin and 1 mM dithiothreitol.

Pretreatment of cells

Four different kinds of cells were prepared for haemolysis: (i) Fresh washed oxygenated cells; (ii) metabolically depleted washed oxygenated cells; (iii) oxygenated fresh cells in serum; (iv) deoxygenated fresh cells in serum.

For (i) and (ii) the cells were washed three times in 10 volumes of a solution containing 0.145 M NaCl, 5 mM glucose, 5 mM Na_2HPO_4-NaH_2PO_4 (pH 7.4, 37°C) at 0–4°C. The washed packed cells were then centrifuged for 5 min at room temperatures in an Eppendorf type 3200 centrifuge to give samples with haematocrit values $\geqslant 97\%$. The packed cells were equilibrated at 37°C for 10 min before haemolysis.

For (iii) and (iv) the defibrinated blood was centrifuged at room temperature for 5 min at $1000 \times g$ under an atmosphere of either 5% CO_2/95% O_2 for oxygenated cells or 5% CO_2/95% N_2 for deoxygenated cells. The packed cells were then centrifuged as described above to give samples with haematocrit values $\geqslant 94\%$. These packed cells were then transferred to a round bottom flask and equilibrated at room temperature with the relevant gas mixture for 10 min. Swirling the flask with gas passing through facilitated the oxygenation/deoxygenation. The cells were kept in the flask with gas flowing through for another 10 min at

37°C before haemolysis, and all subsequent operations were done under the same gas mixtures.

Preparation of the haemolysates

Two methods were used to haemolyse the packed cells: saponin treatment, and freezing followed by thawing at 37°C. In the former case, small aliquots of saponin solution (20 w/v% in 0.15 M NaCl) were added to the packed cells at 37°C. Freezing was done either rapidly using liquid N_2, or more slowly in an ice-NaCl mixture at −10°C. Complete haemolysis was checked for by taking an aliquot of the lysate, suspending it in 0.15 M NaCl, and centrifuging it for a few seconds in the Eppendorf centrifuge. Incomplete haemolysis was then readily apparent by a pellet of intact cells at the bottom of the tube. As will become clear later, incomplete haemolysis should not in principle affect our results. However, we did not want to obtain filtrates that represented only the cytoplasm of cells that were more susceptible to haemolysis. Therefore, we added saponin or continued with the freeze-thawing procedure until > 90% haemolysis was achieved. For fresh washed cells either three cycles of freeze-thawing or 0.1% saponin in the lysate was sufficient. However, the metabolically depleted cells and fresh cells in their plasma were very resistant to haemolysis. This effect has been noted before for stored erythrocytes subjected to saponin induced haemolysis [14]. We found that we needed up to 0.55% saponin in the lysate, and up to 8 cycles of freezing and thawing to achieve complete haemolysis for the more resistant cells. The lysates were kept at 37°C for 5 min before filtration.

After filtration, the pH (and for the deoxygenated lysates, pO_2) of the total haemolysate was measured. The pH was 7.3–7.4, and 7.4–7.5 for the washed cell and unwashed cell lysates, respectively. For deoxygenated lysates pO_2 was $\leqslant 10$ torr, so the Hb was more than 90% deoxyHb [15].

Filtration of the haemolysates

Filtration was done at 37°C. For lysates equilibrated with 5% CO_2 the filtration was carried out in a controlled atmosphere bag containing the relevant gas.

A number of filtration methods were tried and assessed for efficient removal of erythrocyte membranes from the filtrate and minimal loss of enzymes by adsorption to the filters. The method we finally adopted based on these criteria was as follows: The lysate was drawn into a 2 ml glass silicon greased syringe with luer-lock fitting. The syringe was then connected to a Millipore Swinnex holder containing a stack of filters: two 3 μm pore filters at the top (nearest the syringe), followed by two 0.8 μm pore filters, and two 0.45 μm pore filters at the bottom. The syringe with filter assembly was held vertically, and the plunger of the syringe pushed down until the bottom filter was covered with filtrate. (This was seen by holding the filter assembly over a mirror). The syringe was then inverted, and the plunger drawn back. The Swinnex holder was dismantled and all the filters removed except the bottom one. Another top section of a Swinnex holder containing a 3 μm filter was then screwed down over this filter and its support, and the filtrate that had been trapped on the bottom filter washed out with the enzyme stabilizing solution. (The 3 μm filter aided uniform washing of the bottom filter). The unfiltered haemolysate was quantitatively diluted into the enzyme stabilizing solution to give approximately the same concentration of cytoplasm as the diluted filtrate.

Erythrocyte membrane concentrations in the diluted filtrates were measured by assaying the activity of the membrane-bound enzyme, acetylcholinesterase. The rather complicated filtration protocol described above was used because we found that efficient removal of membranes from the filtrate was only possible if the filtrate volume was kept small (< 60 μl). Such a small amount of viscous cytoplasm spread on the bottom filter and filter support precluded an accurate quantitative dilution of the filtrate into the enzyme stabilising solution. To calculate the amount of membrane-bound glyceraldehyde-3-phosphate dehydrogenase we used the relative amounts of cytoplasmic components (in filtrate and total haemolysate) that are not membrane bound to calculate how much glyceraldehyde-3-phosphate would be expected in the filtrate relative to the total haemolysate if none of the enzyme was membrane-bound. Any reduction from this value was then attributed to enzyme binding to the cell membranes. Initially, we used

both K^+ and lactate dehydrogenase (LDH) as the cytoplasmic components that show no affinity for the cell membranes, and calculated free glyceraldehyde-3-phosphate dehydrogenase (G3PDH) as

$(R_{G3PDH}/R_{LDH})\,100$ and $(R_{G3PDH}/R_K)\,100$

where R = ratio of enzyme activity (or K^+ concentration) in the filtrate to that in the total haemolysate.

Validation of the filtration procedure

This method of assessing glyceraldehyde-3-phosphate dehydrogenase binding to the cell membranes is only valid if the filters themselves do not adsorb the enzymes and K^+. To check for this we prepared membrane free haemolysates and filtered them in the same way as haemolysates containing membranes. The membrane-free lysates were made by haemolysing packed washed metabolically depleted cells with saponin (0.55 w/v% in the lysate), and centrifuging 1-ml aliquots for 9 h at 4°C (Beckman L2-65B Ultracentrifuge, SW50.1 head, 4 ml tubes at 45 000 rev./min (186 000 × g_{max})).

Results and Discussion

Validation of the filtration procedure

Saponin increases the buoyant density of plasma membrane [16]. Thus when packed cell haemolysates (1 ml per tube) were centrifuged the membranes sedimented to the bottom of the tube. K^+ was found to be distributed uniformly, and the free cytoplasmic enzymes and Hb sedimented to a degree determined by their effective molecular weights. 0.55-ml aliquots were taken from the top of each tube, mixed well, and found to contain 60–65% of the lactate dehydrogenase, glyceraldehyde-3-phosphate dehydrogenase and aldolase activity per ml, and < 0.3% of the acetylcholinesterase activity per ml compared to uncentrifuged controls.

Table I shows the results of two experiments when such membrane free haemolysates prepared from metabolically depleted cells were filtered. It should be pointed out that the activity ratios, R, reflect not only how the filters have altered the filtrate but also the relative dilutions of filtrate and filtrant into the enzyme stabilizing solution. If the filtration has not altered the composition of the lysate then all R values will be the same, and any difference from $R = 1$ merely indicates the different dilutions of filtrate and filtrant.

TABLE I

FILTRATION OF MEMBRANE FREE HAEMOLYSATES

Data are shown for two experiments. Membrane-free haemolysates, prepared from packed cells of haematocrit, H, were filtered as described in the text. R for each component is the enzyme activity (K^+ concentration) in the filtrate divided by that in the unfiltered haemolysate. The filtrate volume was calculated from the K^+ concentration in the filtrate. Haematocrits were measured to a precision of 1.5% by centrifugation for 6 min in a Heraeus Christ Micro-Hämatokrit centrifuge. G3PDH, glyceraldehyde-3-phosphate dehydrogenase; LDH, lactate dehydrogenase.

H	R values				Filtrate volume (μl)
	G3PDH	LDH	K	Aldolase	
0.97	0.60	0.60	0.65	0.30	55
0.98	1.12	1.14	1.45	–	20

The ratio of lactate dehydrogenase and glyceraldehyde-3-phosphate dehydrogenase activities are identical, but significantly less than the K^+ concentration ratios. Therefore, the filters bind some of the enzymes, and we cannot use R_K to assess enzyme binding to the membranes. Fortunately, the extent of enzyme adsorption to the filters is the same for lactate dehydrogenase and glyceraldehyde-3-phosphate dehydrogenase. In our previous work [8] we could find no evidence for lactate dehydrogenase binding to the red cell membrane even at low ionic strength. We therefore feel justified in using lactate dehydrogenase as a free cytoplasmic marker for calculating the % free glyceraldehyde-3-phosphate dehydrogenase. It will be noticed that the smaller the volume of filtrate the greater the discrepancy between $R_{LDH(G3PDH)}$ and R_K. This is because once the filters have become saturated with bound enzyme further filtration yields a filtrate of identical composition to the lysate.

It is clear from Table I that aldolase adsorption to the filters is much greater than that of lactate dehydrogenase. This explains our finding of ap-

parent aldolase binding to membranes when we filtered total haemolysates (data not shown). Unfortunately, we have not got a free cytoplasmic component that behaves the same as aldolase, so this method cannot be used for studying aldolase binding to erythrocyte membranes. We have also tried using fewer filters, and a Millipore prefilter. In this case, the binding of lactate dehydrogenase and glyceraldehyde-3-phosphate dehydrogenase to the prefilter was still comparable but much larger than to the filters alone (data not shown).

Glyceraldehyde-3-phosphate dehydrogenase binding to erythrocyte membranes

When we came to filter haemolysates containing membranes we rejected all experiments where the volume of filtrate was $< 20\ \mu l$. Smaller volumes sometimes gave $R_{G3PDH} < R_{LDH}$ by up to 13%. However, we do not believe this reflects binding of glyceraldehyde-3-phosphate dehydrogenase to the erythrocyte membranes, but a slightly greater affinity of glyceraldehyde-3-phosphate dehydrogenase compared to lactate dehydrogenase for the filters. When we increased the volume of filtrate so $R_{LDH(G3PDH)} = R_K$ the filtrates were often heavily contaminated with membranes.

Table II summarises the results of the filtration experiments. Within experimental error ($\pm 5\%$) there is no detectable binding of glyceraldehyde-3-phosphate dehydrogenase to the erythrocyte membranes. The factors of relevance to our experiments that influence glyceraldehyde-3-phosphate dehydrogenase binding are the state of oxygenation of Hb and the concentration of anionic metabolites. It has recently been shown [17] that when Hb concentrations are sufficiently high so there is little tendency for Hb tetramers to dissociate into dimers, deoxyHb shows a greater affinity for the Band 3 cytoplasmic domain on the erythrocyte membrane than oxyHb. Glyceraldehyde-3-phosphate dehydrogenase competes for this same binding site [18], and is displaced from it by anionic metabolites [1]. Therefore, the oxygenated metabolically depleted cells should yield lysates with the optimum chance of detecting glyceraldehyde-3-phosphate dehydrogenase binding. Even in this cell preparation we could detect no significant binding.

Assuming the cellular Band 3 and glyceraldehyde-3-phosphate dehydrogenase concentrations are 36 μM and 9 μM, respectively [7], our results suggest the maximum value of the apparent association constant, K_a, of the glyceraldehyde-3-phosphate dehydrogenase-Band 3 complex under phys-

TABLE II

FILTRATION OF TOTAL HAEMOLYSATES

H and R have the same meanings as in Table I. The % free glyceraldehyde-3-phosphate dehydrogenase was calculated from $(R_{G3PDH}/R_{LDH})100$. The % membranes in the filtrate can be calculated from $(R_{AcChE}/R_K)100$ and overestimated as $(R_{AcChE}/R_{LDH})100$. AcChE, acetylcholinesterase; G3PDH, glyceraldehyde-3-phosphate dehydrogenase; LDH, lactate dehydrogenase.

Cells	H	Haemolysis method	R values				% free G3PDH
			AcChE	G3PDH	LDH	K^+	
Oxygenated metabolically depleted, washed	0.98	saponin 0.5%	0.06	1.14	1.19	1.39	96
	0.97	saponin 0.4%	0.011	0.60	0.57	0.70	105
	0.98	freeze-thaw	0.048	1.04	1.03	1.25	101
Oxygenated fresh washed	0.97	saponin 0.1%	0.072	0.97	0.96	1.00	101
	0.97	saponin 0.1%	0.037	1.14	1.19	1.20	96
	0.98	freeze-thaw	0.050	0.85	0.88	1.06	97
Oxygenated fresh unwashed	0.97	saponin 0.53%	0.004	0.87	0.86	–	101
	0.96	freeze-thaw	0.020	1.01	1.04	1.32	97
Deoxygenated fresh unwashed	0.96	saponin 0.33	0.025	1.16	1.12	–	104
	0.94	saponin 0.21%	0.039	1.12	1.13	–	99

iological conditions is about 10^{-3} M^{-1}. This is an order of magnitude smaller than the value obtained by Kliman and Steck [7], who lysed cells at a maximum 10% haematocrit. Given the long extrapolation of their data to the concentrated lysates used in our experiments, our low K_a value is perhaps not unreasonable, reflecting not only the ionic nature of the glyceraldehyde-3-phosphate dehydrogenase-interaction [1], but also inhibition of glyceraldehyde-3-phosphate dehydrogenase binding by other proteins [2,3,17] and polyanions [7] present in the minimally diluted cytoplasm.

Our conclusion that in situ glyceraldehyde-3-phosphate dehydrogenase is not significantly membrane bound is in agreement with the work of Brindle et al. [19]. Their ^{1}H-NMR study showed that glyceraldehyde-3-phosphate dehydrogenase in intact cells has the same kinetic properties as expected from glyceraldehyde-3-phosphate dehydrogenase free in solution. We conclude that although glyceraldehyde-3-phosphate dehydrogenase binds to Band 3 cytoplasmic sites at low ionic strength, under physiological conditions the interaction is so weak that the amount of bound glyceraldehyde-3-phosphate dehydrogenase is undetectable.

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